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(54) Title: MODULATION OF CALCIUM CHANNEL ACTIVITY

(57) Abstract: An antibody or antibody fragment capable of binding to a protein (TrpC1) that is a novel type of Ca^{2+} channel encoded by a *trp* gene. The protein is a store-operated channel subunit contributing to store-operated Ca^{2+} channels in native mammalian cells, accordingly this represents a potential target for novel drug design.

Modulation of Calcium Channel Activity

The present invention relates to a protein or polypeptide, products and uses thereof, the protein or polypeptide being associated with store-operated Ca^{2+} channel activity. In addition, the present invention provides a novel target site for rationalised drug design.

Background to the Invention

Store-operated channels (SOCs) are plasma membrane Ca^{2+} channels that open when Ca^{2+} levels in sarcoplasmic or endoplasmic reticulum are depleted. SOCs may serve an essential "house keeping" function to refill or reload sarcoplasmic reticulum following Ca^{2+}-release. SOCs are also thought to be involved in muscle contraction, control of cell proliferation and in CD95-mediated cell apoptosis.

It is thought that SOCs are products of the mammalian *trp* gene family (related to *Drosophila* TRP/TRPL genes)[1,2]. It is known from the prior art that expressed *trp3* induces channel activity associated with store-depletion but it requires coactivation of receptors or diacylglycerol[3]. *Trp4* is suggested to be a SOC[4] but is also described as a receptor-operated channel that cannot be activated by store-depletion[5]. In addition, expressed *trp*1 may behave as a SOC[6] but it is also reported to be a basally active channel independent of Ca^{2+} stores[7] and a diacylglycerol-activated channel[8]. It would appear from these studies that heterologous expression of mammalian *trp* genes enhances or generates Ca^{2+} channel activity, but whether any of the genes encode native subunits of store-operated channels or if they are membrane-spanning subunits remains unanswered.

Cardiovascular disease and hypertension are the most common diseases in the western world with high morbidity and mortality rates. One approach to management of such diseases is by modulation of Ca^{2+} channel signals. There is

intense research to develop alternative and more effective cardiovascular /hypertensive therapeutic agents.

Statement of the Invention

In its broadest aspect the present invention provides a protein or polypeptide encoded by a *trp1* nucleic acid, products and uses thereof.

In the present invention a mammalian protein TrpC1 has been investigated and characterised. This protein is smaller than its *Drosophila* TRP or TRPL equivalent, with a shorter C-terminus and, because of its comparative size, this mammalian protein has been previously dismissed as being likely to be a SOC [10]. Surprisingly, our results are at odds with this prediction and we have found that the protein TrpC1 (the mammalian *trp1* gene product) is a SOC in vascular smooth muscle cells. We have been able to detect the TrpC1 protein (encoded by *trp1* gene) in a variety of freshly isolated human, mouse and rabbit cells and have demonstrated that mRNA encoding TrpC1 is broadly expressed in the vasculature.

The present invention also provides a TrpC1-specific antibody targeted to the peptide predicted to contribute to the outer vestibule of the TrpC1 channel. The present invention therefore in another aspect provides a novel target site for the development of new and alternative therapeutics for the treatment of cardiovascular, hypertension and arteriosclerosis and other circulatory diseases in addition it provides a target site for the development of analgesics.

According to a first aspect of the invention there is provided an antibody or antibody fragment capable of binding to a peptide comprising the sequence as set forth in SEQ ID NO:1 or a fragment thereof associated with store-operated Ca^{2+} channel activity.

It will be appreciated that the antibody of the present invention is capable of binding to a sequence consisting essentially of that of SEQ ID NO:1 and that the sequence

may contain a typically conservative, substitution, deletion, addition or mutation but preserve its antigenic site.

Preferably, the antibody is monoclonal or polyclonal alternatively the antibody could be in a recombinant humanised form.

According to a further aspect of the invention there is provided a poly(amino acid), e.g polypeptide, that is capable of binding to the antibody or antibody fragment of the present invention, other than a native protein.

Preferably, the poly(amino acid) is isolated.

Preferably, the poly(amino acid) is no more than 50 amino acids and more preferably is no more than 20 amino acids in length. It is thought that an epitope generally comprises between 6-9 amino acid and it will be appreciated that SEQ ID NO:1 encompasses the TrpC1's epitopic region.

Preferably, the poly(amino acid) consists essentially of the sequence as set forth in SEQ ID NO:1 or a fragment thereof. The sequence may also be a variant thereof, modified by substitution deletion or addition of 1 or more amino acids.

The sequence as set forth in SEQ ID NO:1 is associated with a TrpC1 protein or polypeptide and has Ca^{2+} channel activity in that protein.

Preferably, the poly(amino acid) is encoded by a *trp1* nucleic acid and more preferably the nucleic acid encoding region spans domains S5 and S6 of the *trp1* gene.

Preferably, the poly(amino acid) is of mammalian origin.

According to a yet further aspect of the invention there is provided an antibody or antibody fragment specific for the poly(amino acid) as herein before described.

Results have shown that TrpC1 (as set forth in SEQ ID NO:1) is a novel physiological Ca^{2+} channel subunit in arterial smooth muscle cells. TrpC1 was found in a number of different cell types from a number of different species indicating its widespread potential as a target molecule.

The poly(amino acid) comprises an epitope against which an antibody may be raised; poly(amino acid) may if desired or necessary be conjugated to a hapten for the purposes of raising the antibody.

Further studies have shown that TrpC1 is localised to the plasma membrane and has an extracellular domain. Peptide-specific binding of the antibody had a functional effect, selectively blocking store-operated Ca^{2+} channel activity. The antibody of the present invention is thus a powerful new tool for the study of mammalian *trp1* gene products.

According to a yet further aspect of the invention there is provided use of the antibody of the present invention in identifying a SOC site or Ca^{2+} channel activity.

According to a yet further aspect of the invention there is provided a method of identifying a SOC site or Ca^{2+} channel activity comprising contacting the antibody of the present invention with a tissue or cell sample and detecting or measuring the level of interaction therebetween.

According to a yet further aspect of the invention there is provided a pharmaceutical composition comprising a specific binding partner for the poly(amino acid) of the present invention and a pharmaceutically acceptable adjuvant, diluent or carrier. In another aspect, the composition comprises an antibody or antibody fragment of the present invention and a pharmaceutically acceptable adjuvant, diluent or carrier.

Alternatively the composition may comprise a TrpC1 inhibitor and a pharmaceutically acceptable adjuvant, diluent or carrier.

It will be appreciated that the antibody of the present invention was found to selectively block or inhibit store-operated Ca^{2+} channel activity, accordingly the antibody in itself is of potential therapeutic benefit.

Results have shown that TrpC1 is expressed in dorsal root ganglion and especially in small diameter fibres that are involved in dull pain sensations. Therefore, in one aspect of the invention the pharmaceutical composition may be used for the treatment of pain relief.

A possible candidate blocking agent is 2-APB (2-aminoethoxydiphenyl borate) which we have shown to block "store-operated" calcium entry in arterioles. In one embodiment of the invention the pharmaceutical may comprise a TrpC1 inhibitor in the form of 2-aminoethoxydiphenyl borate or a salt thereof or chemical derivative thereof.

According to a yet further aspect of the invention there is provided use of the protein or polypeptide or antibody of the present invention or a TrpC1 inhibitor for the manufacture of a medicament for the treatment of cardiovascular, hypertensive, arteriosclerotic and other circulatory diseases, or as an analgesic.

In another aspect of the invention the protein or polypeptide or antibody of the present invention or TrpC1 inhibitor can be used for the manufacture of a medicament for the treatment of Alzheimer's disease, vasculature brain disorders and as a neuroprotective agent or as an analgesic.

According to a yet further aspect of the invention there is provided a method of screening therapeutic agents which specifically interact with, and bind to the poly(amino acid) sequence as set forth in SEQ ID NO:1 or a part thereof which

comprises contacting a mammalian cell with a plurality of candidate therapeutic agents, determining those agents which bind to the mammalian cell, and thereby identify agents which specifically interact with and bind to the poly(amino acid) of the present invention.

According to a yet further aspect of the invention there is provided use of the protein or polypeptide of the present invention as a target site for interaction with putative cardiovascular, hypertensive or arteriosclerotic agents or as an analgesic and/or for measuring efficacy of such agents.

The target site of the poly(amino acid) comprises at least a functionally effective part of the sequence as set forth in SEQ ID NO:1 and interaction between a therapeutic agent with this site, so as to block or inhibit expression of the protein or polypeptide, provides an alternative approach to rationalised drug design.

According to a yet further aspect of the invention there is provided a method for the production of a pharmaceutical composition comprising the method of screening as herein before described and furthermore mixing the agent identified, or a derivative or homologue thereof with a pharmaceutically acceptable carrier.

According to a yet further aspect of the invention there is provided a primer comprising the sequence as set forth in SEQ ID NOS: 2 and 3, or parts thereof or homologues thereof, the primer being specific for a nucleic acid encoding the poly(amino acid) of SEQ ID NO:1.

Preferably, the primer may be used to identify *trp1* nucleic acid products.

According to a yet further aspect of the invention there is provided a method of treatment comprising administering to a patient suffering from cardiovascular disease, hypertension, arteriosclerosis or any other circulatory disease or pain a

therapeutically effective amount of the pharmaceutical composition of the present invention.

It will be appreciated that patients suffering from Alzheimer's disease, neurological and brain disorders such as, for example and without limitation strokes or pain, may also be treated by and benefit from the pharmaceutical compositions of the present invention.

Brief Description of the Figures

Fig. 1. TrpC1 expressed in blood vessels. Fig 1(a) mRNA detected by RT-PCR in human LIMA, mouse aorta, and rabbit pial membrane (β-actin and *trp1*), arteriolar fragments and single cell "rings". Predicted sizes of the *trp1* and β-actin PCR products were 423 and 763 bp. Products were not detected if reverse transcriptase was omitted, as shown for rabbit pial (no RT). (b) Rabbit arteriolar smooth muscle "ring" stained with anti-α-SMA-Cy3. Scale bar 50μm. (c) Western blots with T1E3 antibody for aorta, LIMA, portal vein (PV) and pial membrane. Labelling was competed off by peptide in human vessels and mouse pial membrane (not shown) and PV (shown).

Fig. 2. shows the putative pore region of SEQ ID NO:1 and targeting of T1E3 antibody to the outer vestibule of TrpC1 channels. Sequence alignment of putative pore regions of *trp* gene products between 5th and 6th membrane spanning domains (S5 and S6), SEQ ID NOS: 6-15. Accession numbers are (r = rat, m = mouse, h = human, o = rabbit): AF061266 (rTrpC1); U73625 (mTrpC1); AF170493 (oTrpC1); U31110 (hTrpC1); AF111107 (mTrpC2); U47050 (hTrpC3); AF170456 (hTrpC4); AF029983 (mTrpC5); AF080394 (hTrpC6); AF139923 (mTrpC7).

Fig. 3. Shows TrpC1 expression in native cells and spanning of the plasma membrane. Fig 3 shows (a) Permeabilised mouse pial arteriole labelled with T1E3 antibody. The edge of one smooth muscle cell is indicated with an arrow. Figure 3

(b) shows another mouse arteriole stained with anti-α-SMA-Cy3. The scale bar is 50μm and applies to images in Figures 3(a), (b), (c) and (d). Fig 3(c) FITC fluorescence from the same arteriole as in Fig 3 (b), which had also been incubated with T1E3 preadsorbed to antigenic peptide. Fig 3 (d) Permeabilised cultured human LIMA smooth muscle cell double-labelled with T1E3 antibody (green; appearing as white to light grey in a black and white image) and anti-α-SMA-Cy3 (red; appearing as dark grey in a black and white image). (e) Live-cell staining of an enzymatically-isolated rabbit arteriole stained with T1E3 antibody. Two smooth muscle cells are in the focal plane and fluorescence is observed only at the perimeter of each cell. The scale bar in (e) is 5μm.

Fig. 4. Shows TrpC1 is a store-operated Ca^{2+} channel. Fig 4 (a) La^{3+}-sensitive Ca^{2+}-entry in a rabbit arteriolar cell after 1 hr in thapsigargin (1μmole/L) (store-depleted). Fig 4 (b) Stores were depleted and the Ca^{2+} signal occurring on reintroduction of 1.5mmole/L Ca^{2+} (as in Fig 4 (a)) was smaller with T1E3 compared against T1E3 plus peptide (n=95 each, * P<0.05). Without store-depletion there was no effect of T1E3 (n=70 each). Nω-nitro-L-arginine methyl ester (0.3mmole/L) was included in Fig 4 (a) and Fig 4 (b) to inhibit basal nitric oxide production. Fig 4 (c) as in Fig 4 a), stores were depleted. The signal occurred on addition of 10mmole/L Ba^{2+} (200 s) and data are mean±s.e.m. (n=55 for each). The signal was smaller with T1E3 compared against T1E3 plus peptide (** P<0.001 at 800 s).

Fig 5 shows distribution analysis of TrpC1 protein in various tissues from a cynomologous macaque primate. Test images are from experiments using a primary antibody, either the T1E3 antibody or an Anti-Trpc1 antibody (purchased from Alomone Labs) and control images are from parallel experiments without a primary antibody. Nuclei are stained blue with haematoxylin in all images (appearing as black in black and white images). Primary antibody binding is indicated by brown colouration (appearing as black to grey in test versus control images). Figure 5A shows the distribution in heart artery and myocardium stained using the Vector Laboratories Vectastain Universal ABC kit and the Anti-Trpc1 antibody, Figure 5B is

the parallel control. Figure 5C shows distribution in heart artery and myocardium stained using the Vector Laboratories Vectastain Universal ABC kit and the T1E3 antibody, Figure 5D is the parallel control. Figure 5E shows the distribution in dorsal root ganglion using the Vector Laboratories Vectastain Universal ABC kit and the T1E3 antibody, Figure 5F is the parallel control. In Figure 5E arrows indicate small diameter fibres, which were positive for TrpC1 as indicated by binding of T1E3 antibody. Figure 5G shows distribution in occipital cortex artery and parenchyma stained using the Vector Laboratories Vectastain Universal ABC kit and the T1E3 antibody, Figure 5H is the parallel control

Materials and Methods

Human left internal mammary arteries (LIMA) and aortae were obtained with ethical approval. Vessels were placed in Hank's solution (mmole/L): NaCl, 137; KCl, 5·4; $CaCl_2$, 0·01; NaH_2PO_4, 0·34; K_2HPO_4, 0·44; D-glucose, 8; HEPES, 5. Other vessels were from male Wistar rats or BalbC mice. Arteriole fragments were obtained from pial membrane[9]. Cells/vessels were stored at 4°C in Hank's (<13hr). mRNA was isolated with Dynabeads® Oligo $(dT)_{25}$ (Dynal). Bead complexes were washed and transferred to 20µl SuperScript™ reverse transcriptase (Gibco-BRL) at 42°C (30min). Primers for *trp1* were TGGTATGAAGGGTTGGAAGAC (forward) SEQ ID NO:2 and GGTATCATTGCTTTGCTGTTC (reverse) SEQ ID NO:3. Primers for β-actin detection were TTGTAACCAACTGGGACGATATG (forward) SEQ ID NO:4 and GATCTTGATCTTCATGG TGCTGG (reverse) SEQ ID NO:5. Thermal cycling was 95°C (5min), 40 cycles at 94°C (30sec), 53-60°C (1min), 72°C (1min), and 72°C (7min). Products were detected on 1.5% agarose gels and directly sequenced for rabbit pial membrane. T1E3 antibody was prepared in rabbit by Sigma-Genosys (UK) and targeted to TrpC1 of SEQ ID NO:1 (Fig. 2a). Specificity was tested by ELISA and pre-immune serum had no activity. T1E3 antiserum was used at 1:500 dilution and antigenic peptide at 10µmole/L. For experiments in 100mmole/L K^+, antiserum was cleaned on a HiTrap protein A column and used at 1:100 dilution, with/without antigenic peptide at 20µmole/L. For Western blotting,

tissues were placed in 100μmole/L phenylmethylsulphonylfluoride (Sigma) and lysed in SDS buffer containing 100μmole/L dithiothreitol at 80-100°C (15min). Proteins were separated on 10% SDS-PAGE gels and transferred to nitrocellulose, which was rinsed with phosphate buffered saline (PBS) and incubated in PBS containing 10% milk for 1hr (room temperature). Incubation in T1E3 was overnight at 4°C, followed by washes in PBS and incubation in horseradish peroxidase-secondary antibody (1:5000, BioRad) for 1hr (room temperature). Membranes were washed with PBS and labelling detected by ECLplus (Amersham). For immunofluorescence, tissues/cells adhered to poly-L-lysine-coated slides were incubated in 1% BSA/PBS, transferred to T1E3 antibody for 12hr, and secondary antibody (mouse anti-rabbit IgG-FITC, 1:160, Sigma) for 1hr. Cells were identified with anti-smooth muscle α-actin antibody (anti-α-SMA-Cy3, 1:200, Sigma). Microscopy images were processed with Openlab software (Improvision, UK). Permeablised cells were fixed in 2% paraformaldehyde (30min), immersed in –20°C methanol (1min), and 1% BSA with 0.1% triton X-100 for 1hr. Ratiometric $[Ca^{2+}]_i$ or $[Ba^{2+}]_i$ measurements were as described[19] but using 340/380 nm excitation and background fluorescence was subtracted. The superfusion solution contained (mmole/L): NaCl, 130; KCl, 5; $MgCl_2$, 1.2; $CaCl_2$ 1.5; HEPES 10; and glucose 8; pH 7.4; flow rate 5ml/min. Ca^{2+} was replaced by 0.4mmole/L EGTA for Ca^{2+}-free solution. All solutions included methoxyverapamil (10μmole/L). For imaging experiments except those in 100mmole/L K^+ solution, preincubation with 1:500 T1E3 was for 8-12hr (4°C). When 100mmole/L KCl replaced 100mmole/L NaCl in the superfusion solution, arterioles were preincubated with 1:100 cleaned T1E3 for 2hr (37°C). Antiserum/peptide were not in recording solutions. Recordings were made alternately from test and control cells. Signals were measured from 5 smooth muscle cells in each arteriole. Data are expressed as mean± s.e.m. and *n* is the number of cells. Comparisons were made using unpaired Student's *t*-test.

TrpC1 protein in cynomologous macaque primate tissue was investigated using paraffin embedded tissue sections from heart, occipital cortex and dorsal root ganglion. Tissue was stained using the Vector Laboratories Vectastain Universal

ABC kit and either the T1E3 antibody or an Anti-Trpc1 antibody purchased from Alomone Labs.

Evidence in Support of the Invention

EXAMPLE 1

***Trp1* mRNA expression**

Trp1 mRNA expression was investigated in a range of blood vessels using RT-PCR the results indicate that it was present (Fig. 1a). It was also detected in single arteriolar smooth muscle cells harvested by a micro-hooking method (Fig. 1a, b). TrpC1 protein was detected using a polyclonal antibody (T1E3) targeted to a mammalian TrpC1-specific peptide (Fig. 2a). The peptide was predicted to be extracellular based on results of transmembrane detection algorithms (not shown) and studies of TrpC3 glycosylation[11] and *Xenopus laevis trp* expression[12]. Western blotting revealed that T1E3 is specific for protein of the mass predicted for TrpC1 (Fig. 1c), which is 92 kDa for α-splice variant and 87 kDa for β-deletion (human TrpC1). Labelling by T1E3 was peptide-specific because it was absent following preadsorption to antigenic peptide (Fig. 1c). Small variations in the size of labelled proteins may be explained by varying levels of α- and β-variants[6], both of which were detected by RT-PCR.

Membrane-inserted TrpC1 protein was labelled with T1E3 as shown by immunofluorescence staining of permeabilised cells in arterioles (Fig. 3a). Staining was most intense at the edge of smooth muscle cells in arterioles (Fig. 3a) or in cells cultured from human LIMA (Fig 3d), suggesting plasma membrane localisation. Staining was specific because it was absent if T1E3 was preadsorbed to its antigenic peptide (Fig. 3b and 3c). T1E3 antibody should also label unpermeabilised cells if the epitope is extracellular. Smooth muscle cells in enzymatically isolated rabbit pial arterioles were incubated with T1E3 prior to fixation with paraformaldehyde and without Triton-X permeabilisation. Specific staining with T1E3 was detected and was most intense at the cell perimeter (Fig. 3e). The absence of permeabilisation was

confirmed by lack of staining by anti-α-SMA-Cy3 (data not shown). Fluorescence was absent from rabbit arterioles incubated with secondary antibody but not T1E3, or T1E3+peptide (data not shown). Immunofluorescence studies were performed on rabbit as well as mouse because we could not satisfactorily isolate cells from mouse. Isolated rabbit arterioles are amenable to Ca^{2+}-imaging, and we have evidence for SOCs.

EXAMPLE 2

TrpC1 is a store-operated Ca^{2+} channel

Sequence alignments of TrpCs with *Shaker* K^+ channel and related channels suggest TrpCs are channel subunits. These alignments place the T1E3 epitope in the putative outer vestibule of the channel (Fig. 2b). Since antibodies targeted to this region of K^+ channels block K^+-currents we tested whether T1E3 inhibits Ca^{2+} entry. With block of voltage-gated Ca^{2+} channels and after store-depletion caused by thapsigargin, reintroduction of extracellular Ca^{2+} caused a lanthanum-sensitive rise in $[Ca^{2+}]_i$ (Fig. 4a) that was similar to that described previously[13]. The effect of reintroducing Ca^{2+} was significantly larger after thapsigargin treatment (Fig. 4b), suggesting a component of Ca^{2+}-influx through SOCs. To test the effect of T1E3 on SOCs, arterioles were incubated with T1E3 at 4°C to allow binding of antibody but minimise *de novo* protein expression. In thapsigargin treated (but not untreated) arterioles, the $[Ca^{2+}]_i$ signal on reapplication of Ca^{2+} was significantly smaller after incubation with T1E3 as compared to incubation with T1E3 preadsorbed to antigenic peptide (Fig. 4b). Ba^{2+} is permeant in Ca^{2+} channels but is weakly extruded or sequestered by cells. Thus, application of Ba^{2+} may permit a better measure of ion-flux through SOCs. Ba^{2+}-influx was measured after thapsigargin treatment and was significantly smaller following incubation in T1E3 without antigenic peptide (Fig. 4c). The effect of T1E3 did not result from an effect on membrane potential because T1E3 also inhibited Ba^{2+}-influx when arterioles were studied in solution containing 100mmole/L K^+, which strongly depolarises and clamps the membrane potential

(data not shown). In this condition, Ba^{2+}-induced $\Delta F_{340}/F_{380}$ was again significantly smaller in the T1E3 compared with the T1E3+peptide group (0.1728±0.0054 n=50 *vs* 0.2153±0.0112 n=40, P<0.0005). Antigenic peptide alone had no effect on Ba^{2+}-flux: $\Delta F_{340}/F_{380}$ was 0.219±0.007 in control and 0.211±0.008 in peptide (n=75 for each, P>0.05).

That SERCA inhibition increased the $Ca^{2+}{}_i$ signal on reintroduction of extracellular Ca^{2+} is suggestive of SOC activity, although the effect could be explained by the superficial buffer barrier hypothesis with constant background Ca^{2+} entry. We now show the effect of T1E3 on background Ca^{2+} signal alone and the signal plus that induced by SERCA inhibition. Importantly, T1E3-sensitivity depended on thapsigargin treatment. Thus, TrpC1 is not a background Ca^{2+} channel but a Ca^{2+} channel activated by store-depletion.

EXAMPLE 3

TrpC1 is a plasma membrane protein spanning the membrane with an extracellular domain

Three observations demonstrate TrpC1 is a plasma membrane protein spanning the membrane with an extracellular domain. T1E3-labelling is most intense at the cell perimeter. T1E3 labelled cells that were not permeabilised. Incubation of live cells with T1E3 inhibited Ca^{2+} entry. In the latter two cases, T1E3 must have bound an extracellular site. The Ca^{2+}/Ba^{2+} measurements further suggest TrpC1 is a pore-forming subunit because the T1E3 epitope is in the predicted outer vestibule of the channel. Although the blocking effect of T1E3 might seem relatively small, the effect was statistically significant in three independent data sets. Furthermore, a large block was not expected: 1. Only part of the Ca^{2+}/Ba^{2+}-influx was store-operated. 2. A large antibody molecule is unlikely to be an efficient channel blocker. 3. We incubated with T1E3 for relatively short periods (8-12hr at 4°C or 2hr at 37°C) to minimise changes to native protein levels or cellular localisation. Although T1E3

was washed out prior to Ca^{2+}-measurements it remained bound as demonstrated by immunostaining and Western blot.

These results have led us to believe that TrpC1 is one pore-forming subunit in a SOC heteromultimer, another subunit having a longer C-terminus and that there is more than one mechanism by which SOCs can couple to Ca^{2+} stores and the mechanism involving TrpC1 is different from that involving *Drosophila* TRP.

The present invention therefore provides a protein (TrpC1) that is a novel type of Ca^{2+} channel in mammalian vascular smooth muscle encoded by a *trp* gene. The protein of the present invention is of particular advantage in that it is a native store-operated channel. In addition, the antibody of the present invention is of particular utility as a tool for studying TrpC1 effects and demonstrates that the *trp*1 gene encodes a novel channel subunit contributing to store-operated Ca^{2+} channels in native arterial smooth muscle cells. Moreover, TrpC1 is a potential target for novel drugs to alleviate hypertension or vasospasm, or inhibit smooth muscle proliferation in arteriosclerosis and neointimal hyperplasia.

EXAMPLE 4

Differential expression of TrpC1

With regard to Figure 5 we have demonstrated distribution analysis of TrpC1 protein in cynomologous macaque primate tissue: occipital cortex, heart, and dorsal root ganglion. The data indicate differential expression of TrpC1 and that TrpC1 is expressed at highest levels in cardiac muscle cells, dorsal root ganglion neurones (especially small fibres) and cerebral arteries. TrpC1 is expressed in cerebral artery and myocardium but not coronary artery. We have also observed that TrpC1 is expressed in dorsal root ganglion and especially in small diameter fibres that are involved in dull pain sensations.

Differential expression suggests some specificity could be achieved through use of a TrpC1 blocking substance. The function of TrpC1 in cardiac muscle is unknown and it is difficult to speculate on what would happen if TrpC1 was blocked in the heart. The TrpC1 expression in small sensory neurone fibres of the dorsal horn is we believe a possible target site for developing blockers of TrpC1 that may have use as analgesics. A possible candidate blocking agent is 2-APB (2-aminoethoxydiphenyl borate) which is known to block "store-operated" calcium entry in arterioles.

Results also suggest that TrpC1 mRNA levels may increase in proliferating vascular smooth muscle and that a small molecule inhibitor of the TrpC1 calcium signal inhibits phenotypic modulation. These data appear to support a hypothesis that a TrpC1 blocker would be beneficial in arteriosclerosis and neoinitimal hyperplasia.

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Claims

1. An antibody or antibody fragment capable of binding to a peptide comprising the sequence as set forth in SEQ ID NO:1 or a fragment thereof.

2. An antibody or antibody fragment according to claim 1 which is monoclonal or polyclonal.

3. An antibody or antibody fragment according to claim 1 which is in a recombinant humanised form.

4. A poly(amino acid) that is capable of binding to the antibody or antibody fragment according to any preceding claim.

5. A poly(amino acid) according to claim 4 which is isolated.

6. A poly(amino acid) according to either claim 4 or 5 comprising no more than 50 amino acids.

7. A poly(amino acid) according to claim 6 comprising no more than 20 amino acids.

8. A poly(amino acid) according to any one of claims 4-7 consisting essentially of the sequence as set forth in SEQ ID NO:1 or a fragment thereof.

9. A poly(amino acid) according to any one of claims 4-7 wherein the sequence is a variant of a poly(amino acid) of claim 8, modified by substitution deletion or addition of 1 or more amino acids.

10. An antibody or antibody fragment specific for the poly(amino acid) according to any one of claims 4-9.

11. Use of an antibody or antibody fragment according to any one of claims 1-3 or 10 in identifying a SOC site or Ca^{2+} channel activity.

12. A method of identifying a SOC site or Ca^{2+} channel activity comprising contacting the antibody or antibody fragment according to any one of claims 1-3 or 10 with a tissue or cell sample and detecting or measuring the level of interaction therebetween.

13. A pharmaceutical composition comprising a specific binding partner for the poly(amino acid) according to any one of claims 4-9 and a pharmaceutically acceptable adjuvant, diluent or carrier.

14. A pharmaceutical composition comprising an antibody or antibody fragment according to any one of claims 1-3 or 10 and a pharmaceutically acceptable adjuvant, diluent or carrier.

15. A pharmaceutical composition comprising a TrpC1 inhibitor and a pharmaceutically acceptable adjuvant, diluent or carrier.

16. A pharmaceutical composition according to claim 15 for the treatment of pain relief.

17. A pharmaceutical composition according to either claim 15 or 16 wherein the TrpC1 inhibitor is 2-aminoethoxydiphenyl borate or a chemical derivative thereof or a salt thereof.

18. Use of a poly(amino acid) according to any one of claims 4-9 for the manufacture of a medicament for the treatment of cardiovascular, hypertensive, arteriosclerotic and other circulatory diseases, Alzheimer's disease, brain disorders or as a neuroprotective agent or as an analgesic.

19. Use of an antibody or antibody fragment according to any one of claims 1-3 or 10 for the manufacture of a medicament for the treatment of cardiovascular, hypertensive, arteriosclerotic and other circulatory diseases, Alzheimer's disease, brain disorders or as a neuroprotective agent or as an analgesic.

20. Use of a TrpC1 protein for the manufacture of a medicament for the treatment of cardiovascular, hypertensive, arteriosclerotic and other circulatory diseases, Alzheimer's disease, brain disorders or as a neuroprotective agent or as an analgesic.

21. Use of a poly(amino acid) according to any one of claims 4-9 as a target site for interaction with putative cardiovascular, hypertensive, arteriosclerotic or neuronal agents and/or for measuring efficacy of such agents.

22. A method of screening therapeutic agents which specifically interact with, and bind to the poly(amino acid) sequence as set forth in SEQ ID NO:1 or a part thereof which comprises contacting a mammalian cell with a plurality of candidate therapeutic agents, determining those agents which bind to the mammalian cell, and thereby identifying agents which specifically interact with and bind to the poly(amino acid) according to any one of claims 4-9.

23. A method for the production of a pharmaceutical composition comprising the method of claim 22 and furthermore mixing the agent identified, or a derivative or homologue thereof with a pharmaceutically acceptable carrier.

24. A primer comprising the sequence as set forth in SEQ ID NOS: 2 and 3, or parts thereof or homologues thereof, the primer being specific for a nucleic acid encoding the poly(amino acid) of SEQ ID NO:1.

25. A method of treatment comprising administering to a patient suffering from cardiovascular disease, hypertension, arteriosclerosis or any other circulatory disease or Alzheimer's disease, neurological or brain disorders or pain, a therapeutically

effective amount of a pharmaceutical composition according to any one of claims 13-17.

FIG 1a

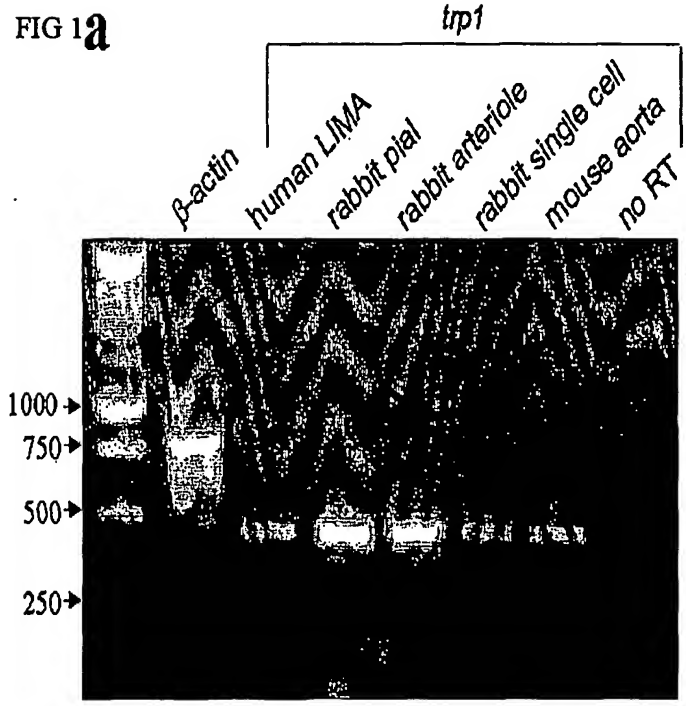

trp1
β-actin
human LIMA
rabbit pial
rabbit arteriole
rabbit single cell
mouse aorta
no RT
1000
750
500
250

FIG 1b

FIGURE 1 C

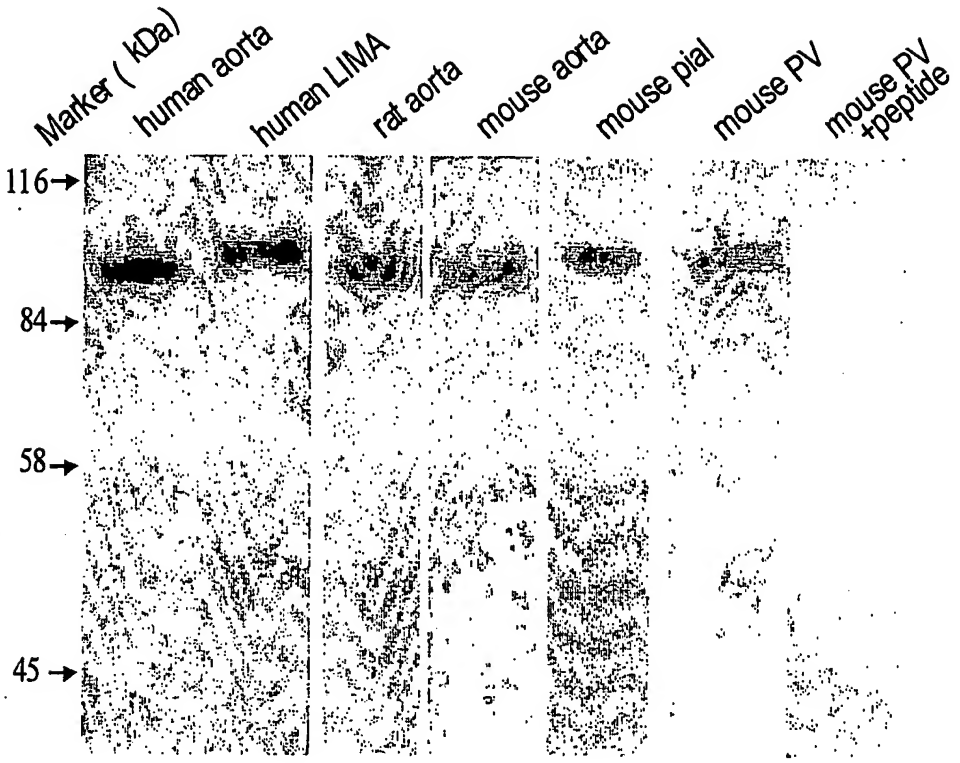

Marker (kDa)
human aorta
human LIMA
rat aorta
mouse aorta
mouse pial
mouse PV
mouse PV +peptide
116
84
58
45

FIGURE 2

SEQ ID NO: 1 CVGIFCEQQSNDTFHSFIGT

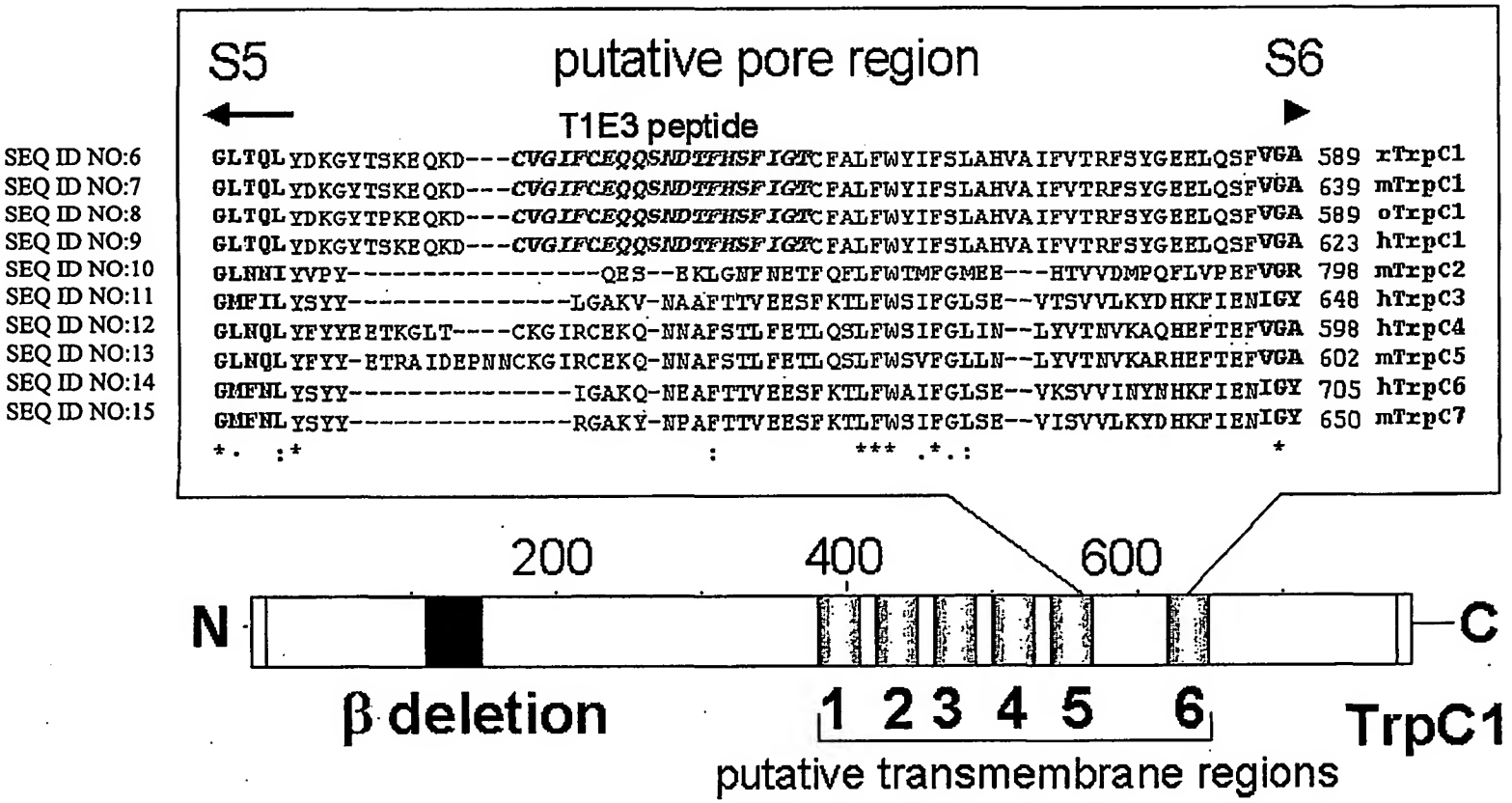


FIGURE 3

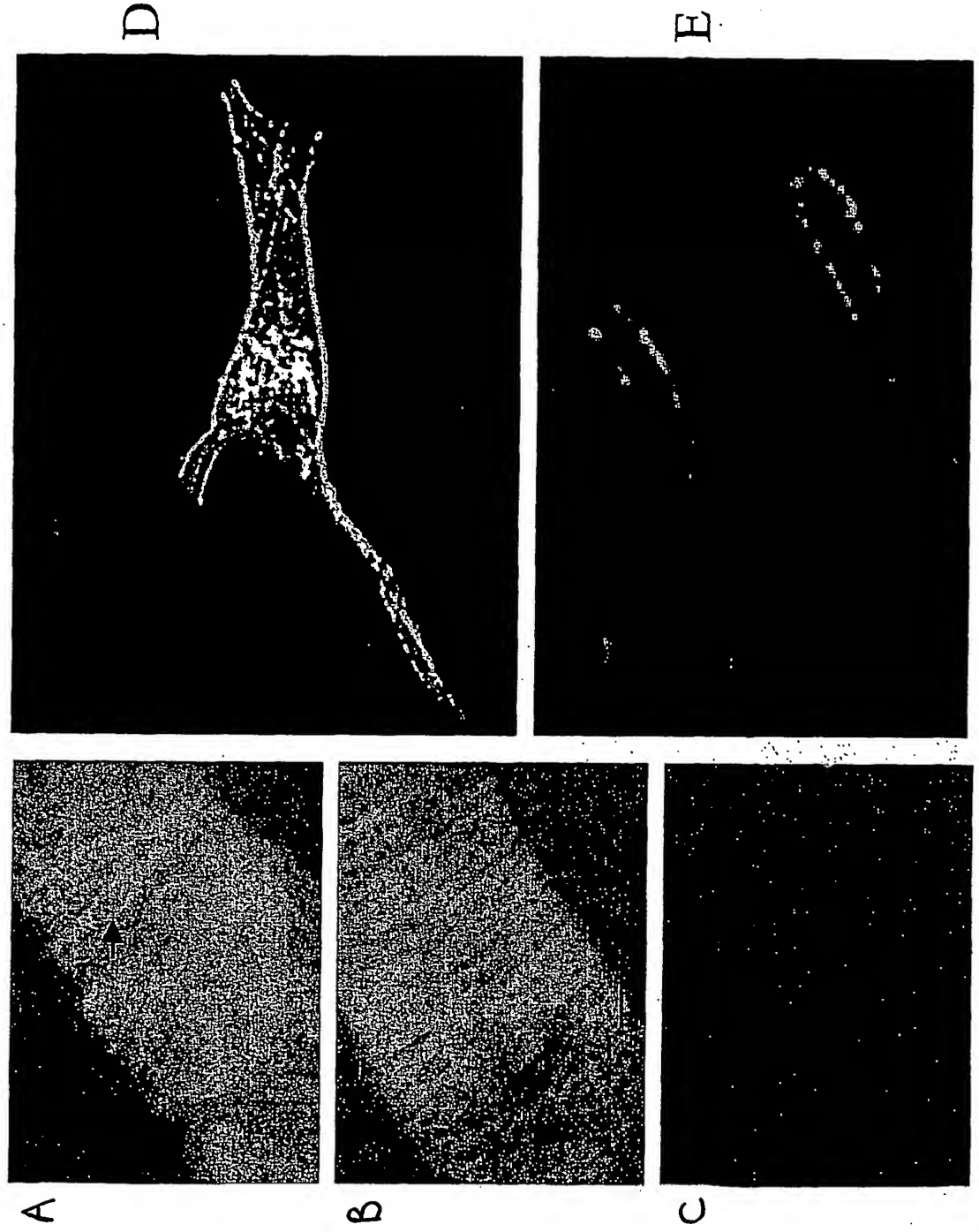


FIGURE 4 A

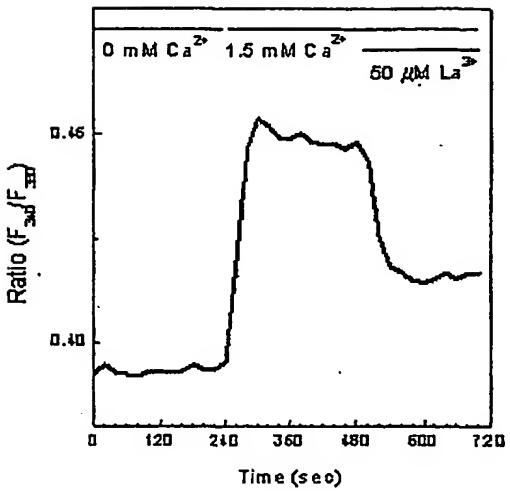


FIGURE 4 B

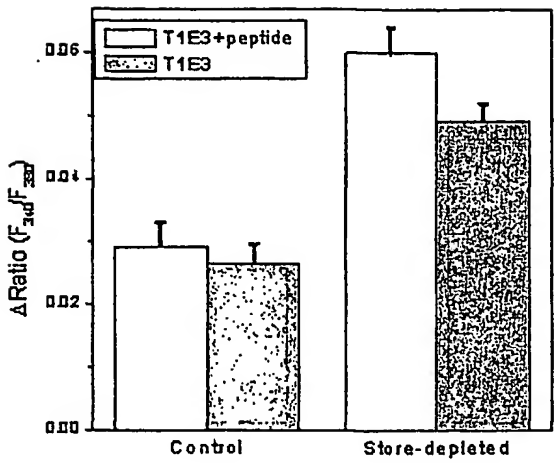


FIGURE 4 C

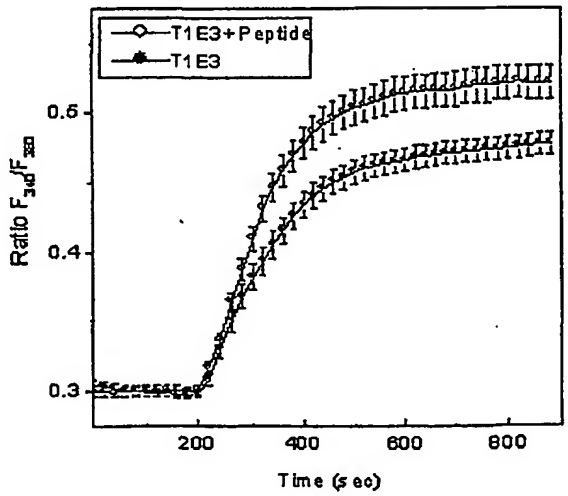


FIGURE 5 A

FIGURE 5 B

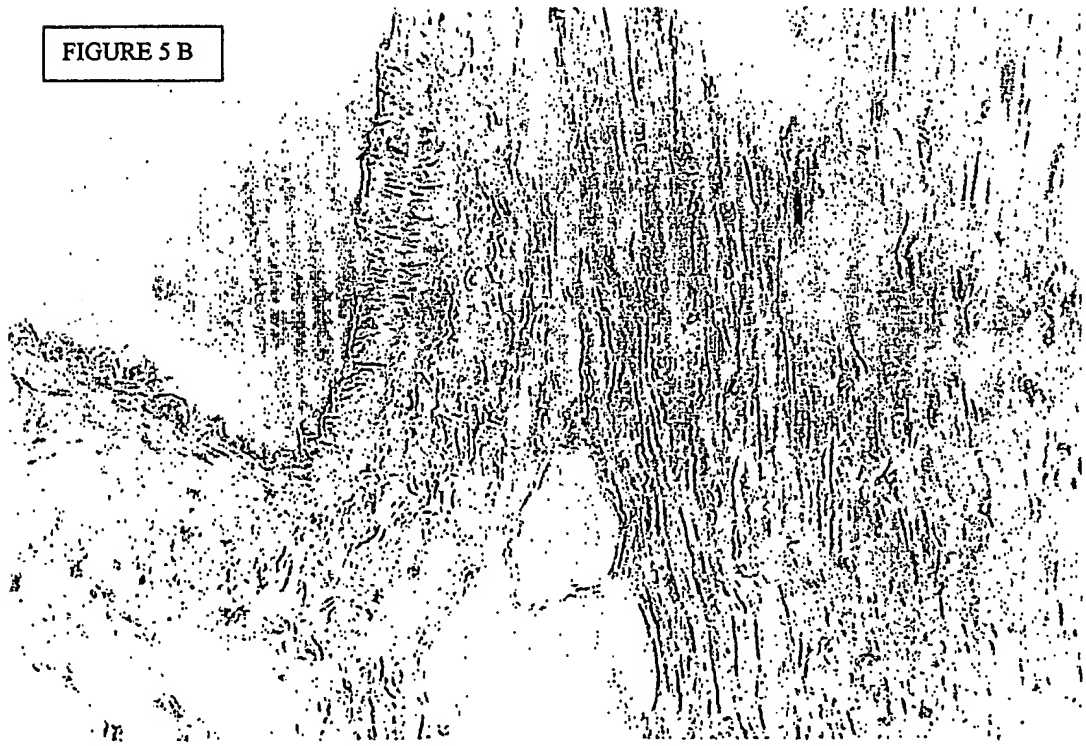


FIGURE 5 C

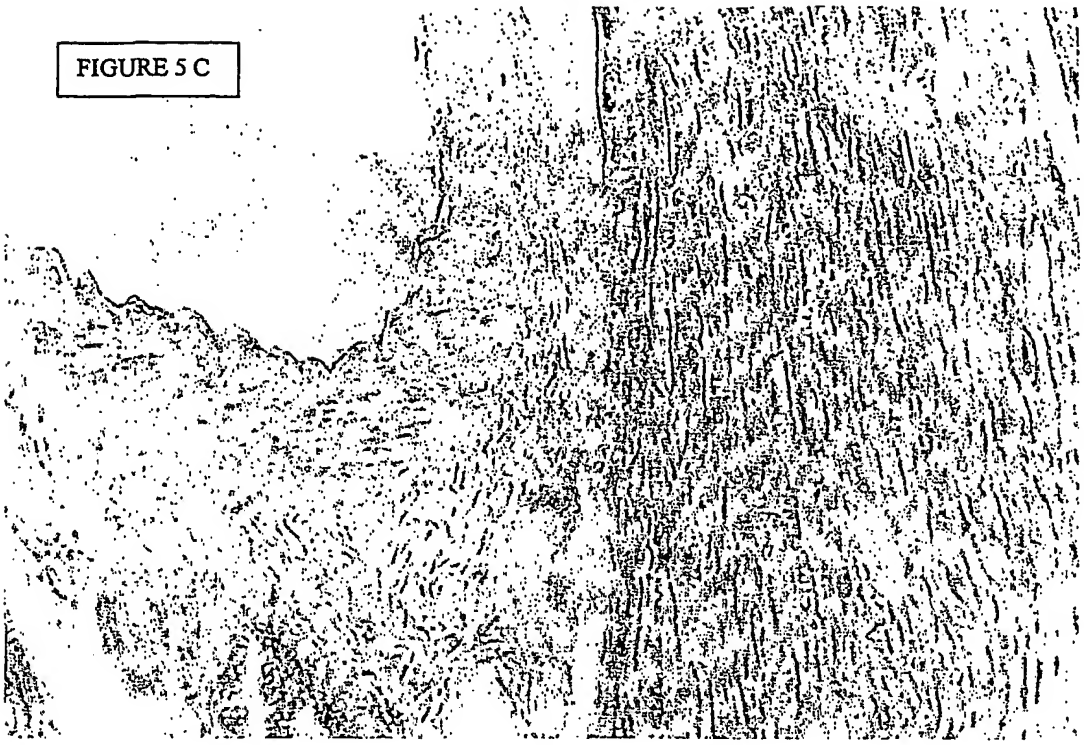


FIGURE 5 D

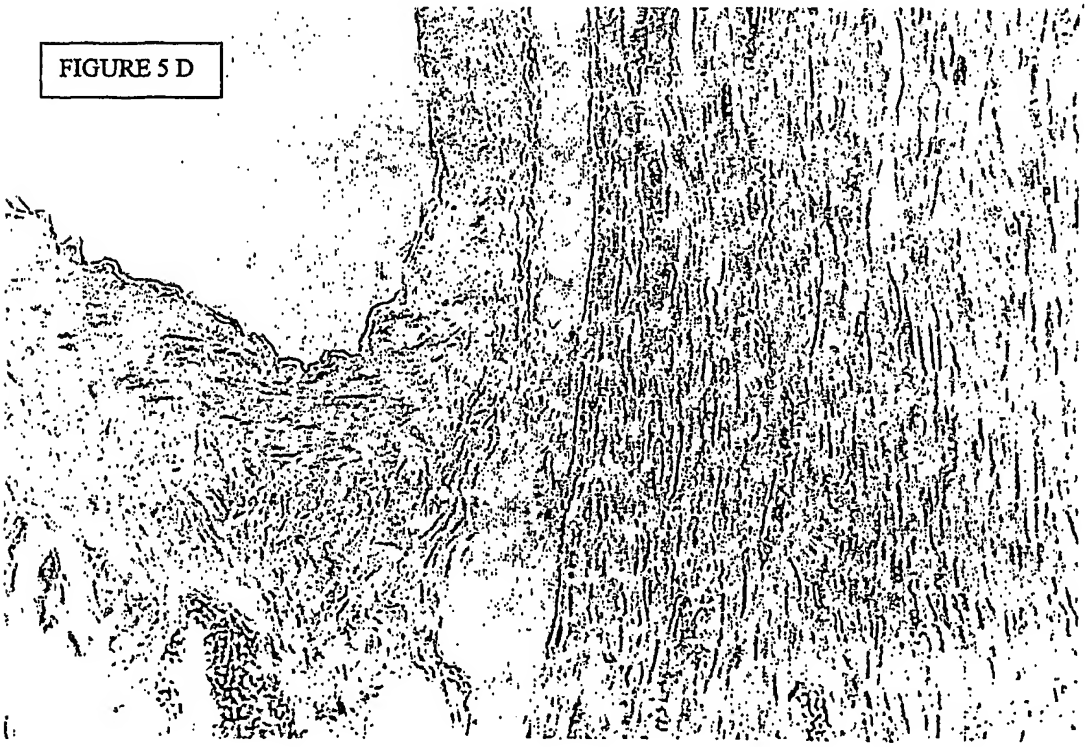


FIGURE 5 E

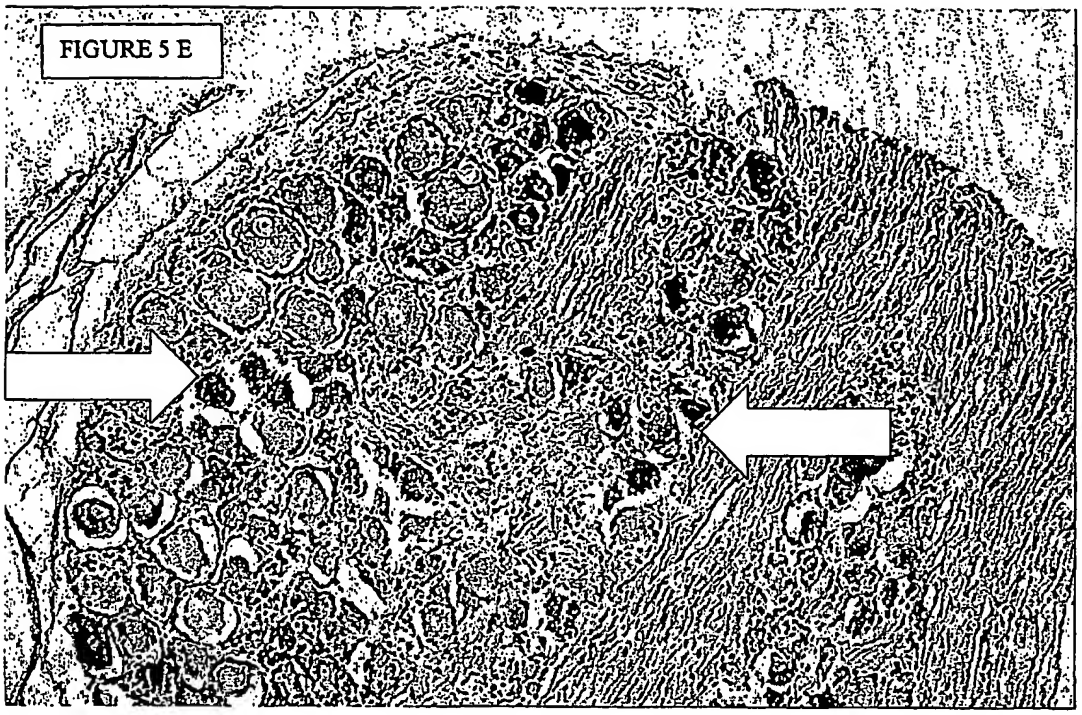


FIGURE 5 F

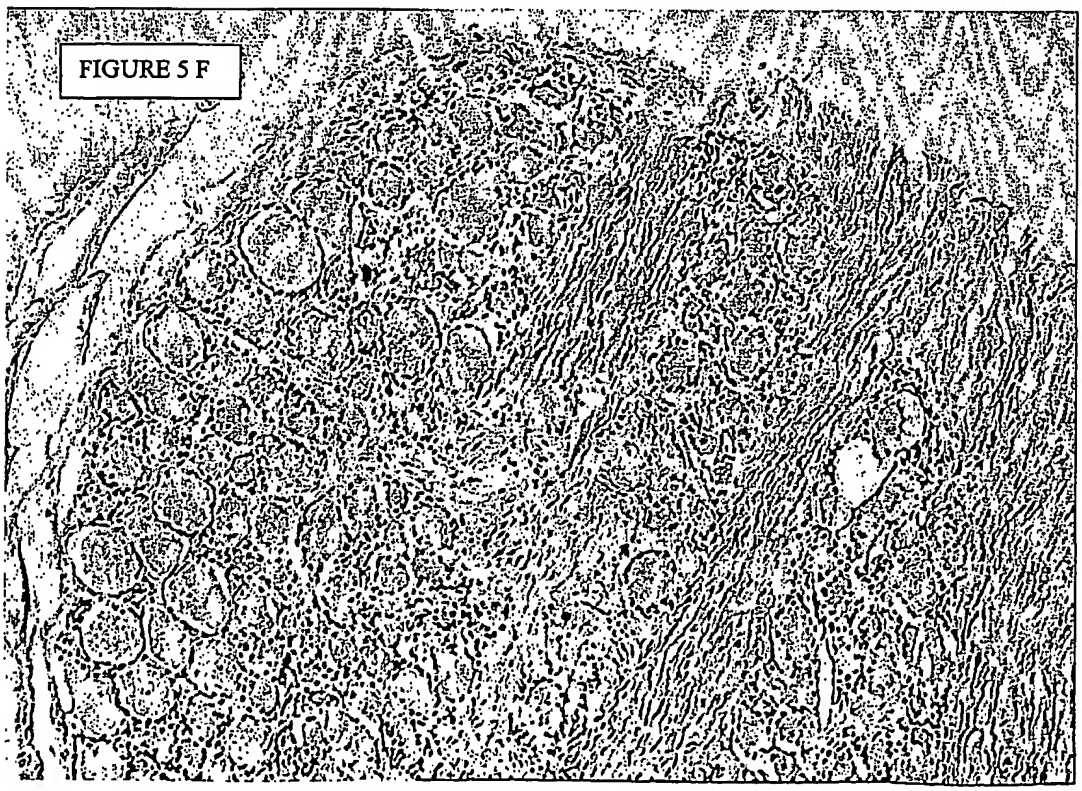


FIGURE 5 G

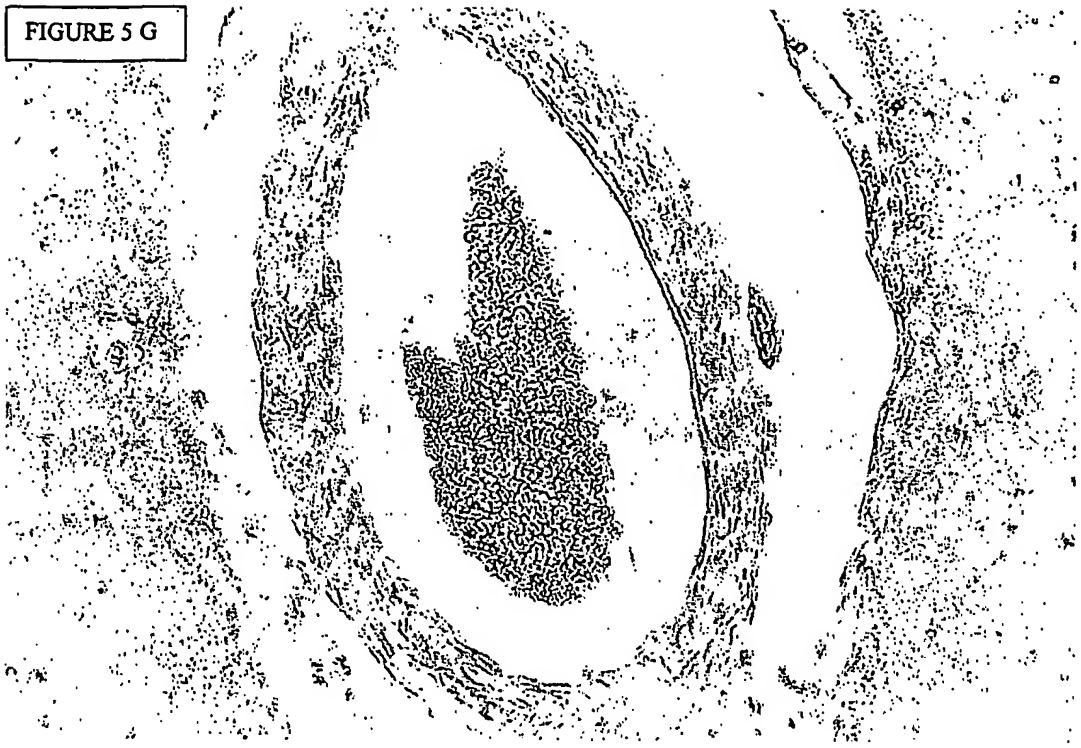
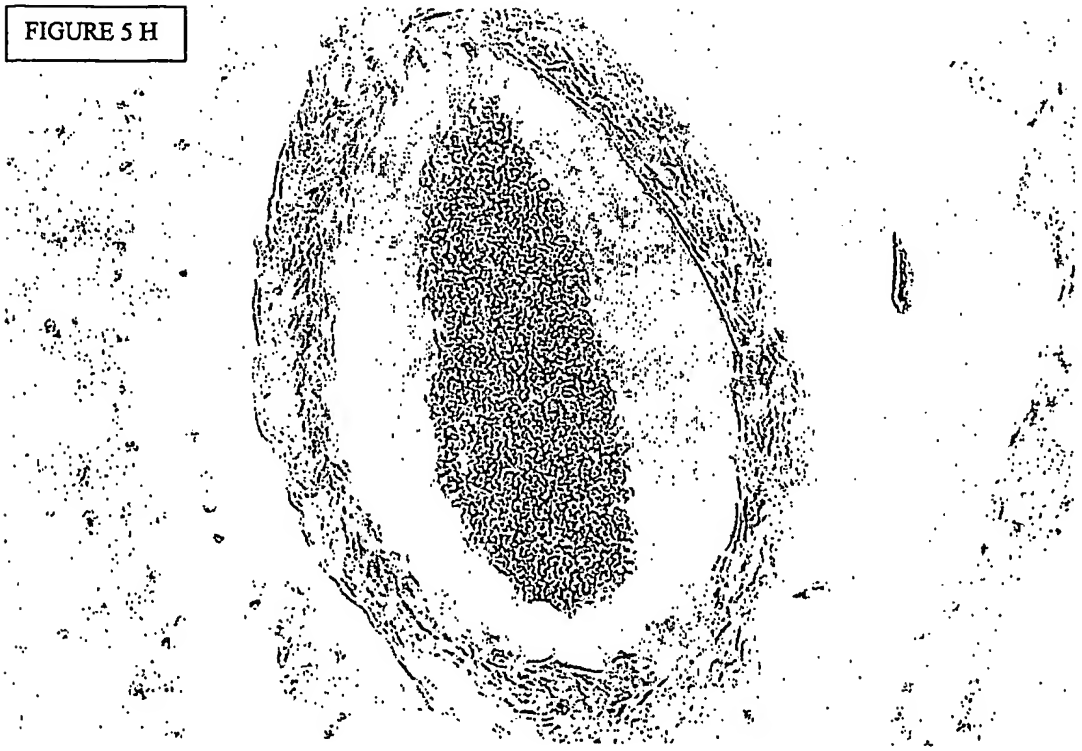


FIGURE 5 H



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Published:
— *with international search report*

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5 December 2002

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 02/059155 A3

(54) Title: MODULATION OF CALCIUM CHANNEL ACTIVITY

(57) Abstract: An antibody or antibody fragment capable of binding to a protein (TrpC1) that is a novel type of Ca^{2+} channel encoded by a *trp* gene. The protein is a store-operated channel subunit contributing to store-operated Ca^{2+} channels in native mammalian cells, accordingly this represents a potential target for novel drug design.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 02/00013

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C07K16/28 C07K14/705 G01N33/577 A61K39/395 A61K31/00
A61P9/00 A61P25/00 A61K38/17 C12N15/12

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
BIOSIS, EMBASE, CHEM ABS Data, WPI Data, PAJ, EPO-Internal, SEQUENCE SEARCH

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	S. XU ET AL.: "TrpC1 is a plasma membrane spanning protein in native arterial smooth muscle cells." JOURNAL OF PHYSIOLOGY, vol. 527P, September 2000 (2000-09), page 80P XP008007039 Cambridge, GB the whole document ---	1,2,4,5, 8,10-12, 22
A	P. WES ET AL.: "TRPC1, a human homolog of a Drosophila store-operated channel." PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE U.S.A., vol. 92, no. 21, 1995, pages 9652-9656, XP002138820 Washington, DC, USA abstract figure 1 --- -/--	1-25

[X] Further documents are listed in the continuation of box C. [ ] Patent family members are listed in annex.

° Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
29 August 2002	12/09/2002

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Nooij, F

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	D. VAN ROSSUM ET AL.: "Ca2+ entry mediated by store depletion, S-nitrosylation, and TRP3 channels." THE JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 275, no. 37, 15 September 2000 (2000-09-15), pages 28562-28568, XP002211621 Baltimore, MD, USA abstract	15-17
P,X	S. XU ET AL.: "TrpC1 is a membrane-spanning subunit of store-operated Ca(2+) channels in native vascular smooth muscle cells." CIRCULATION RESEARCH, vol. 88, no. 1, 19 January 2001 (2001-01-19), pages 84-87, XP008007042 Dallas, TX, USA the whole document	1-15, 18-25

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1-14, 18-24 (completely), 25 (partially)

   Poly(amino acid) or fragment or derivative thereof, consisting essentially of SEQ ID NO:1 or a fragment thereof. Primers specific for a nucleic acid encoding said poly(amino acid). Antibody capabable of binding to said sequence. Methods of screening and therapy, using said poly(amino acid) or antibody.

2. Claims: 15-17 (completely), 25 (partially)

   Pharmaceutical composition comprising a TrpC1 inhibitor, and its therapeutic use.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/GB 02/00013

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claim 25 is directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [X] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.